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"CONSERVATION THROUGH EDUCATION"

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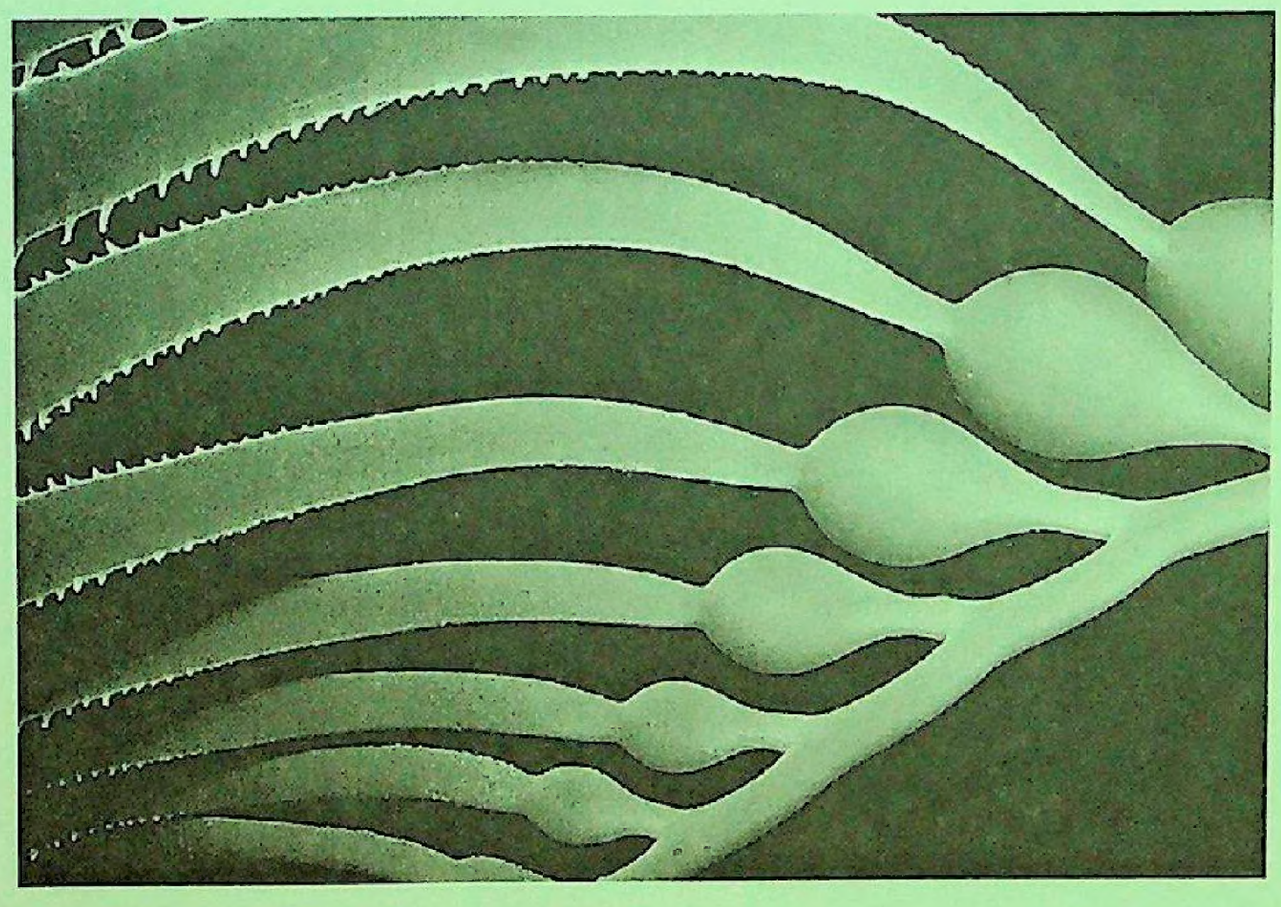

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COVER

Giant Kelp, *Macrocystis pyrifera*

California Fish and Game 84(4):149-158 1998

A NEW TOOL FOR KELP RESTORATION

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We developed new techniques for restoring and protecting giant kelp, *Macrocystis pyrifera*, forests in southern California using artificial kelp plants constructed of plastic. The blades of these artificial plants sweep across the substrate creating a whiplash effect similar to that reported in natural kelp populations in Chile and southern California. The artificial plants reduced the density of purple sea urchins, *Strongylocentrotus purpuratus*, by almost 85% and red sea urchins, *S. franciscanus*, by 75% in sea-urchin-dominated areas. Artificial plants effectively protected giant kelp transplants that were placed in sea-urchin-dominated areas.

INTRODUCTION

Forests of giant kelp, *Macrocystis pyrifera*, occur in California from San Mateo County to the Mexican border. These kelp forests are reported to be as complex as terrestrial rain forests (Foster 1975, Dayton et al. 1984, Vasquez 1992) and provide substrate, food, and habitat for a wide variety of marine organisms (Ghilardi 1971, Snider[2] 1985, Vasquez 1993). Forests of giant kelp are also important economically, supporting fisheries for fin fish, invertebrates, and kelp. Forests of giant kelp provide the basis for an alginate industry which has sales exceeding US$230 million annually (Jensen 1993). As much as 155,000 metric tons of kelp have been harvested annually in southern California (McPeak and Barilotti 1993).

Sea urchins that live in forests of giant kelp typically feed on kelp debris that drifts down from the forest (Harrold and Reed 1985). Well-fed sea urchins that are receiving ample drift kelp move very little (Foster and Schiel 1985). When sea urchins become abundant they can have devastating effects on populations of marine plants, especially various kelps and other brown algae. Such effects have been noted in algal communities over a broad geographical range (see reviews by Lawrence 1975, Harrold and Pearse 1987, Vasquez and Buschmann 1997). Grazing is most

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[2] Snider, L.J. 1985. Demersal zooplankton of the giant kelp *Macrocystis pyrifera*: Patterns of emergence and the population structure of three gammarid amphipod species. Ph.D. Dissertation, University of California, San Diego, California, USA.

pronounced when sea urchins form moving aggregations or "fronts" that eat almost all macroalgae in their path, producing "barren grounds" that are devoid of nearly all algae (Lawrence 1975). These sea-urchin-dominated areas can remain barren of algae for many years because some sea urchins remain behind the front and consume newly settled spores and recruited plants. Sea urchins in barren grounds appear to persist primarily on the meager amounts of algae that drift into the area (Mattison et al. 1977, Harrold and Reed 1985), but also maintain themselves by grazing macroalgae (Lawrence and Sammarco 1982), absorbing body tissues (Ebert 1967, Pequignat 1972, De Ridder and Lawrence 1982), or possibly by absorbing dissolved organic matter (Clark[3] 1969, Lawrence 1982).

A kelp restoration project was started off Point Loma, San Diego County in 1963. Since then, kelp restoration techniques have evolved considerably and can be divided into 4 major categories (Wilson et al. 1978): 1) transplanting, 2) monitoring, 3) controlling competitors, and 4) controlling grazers. Through the mid-1980s, the project concentrated on grazer control through the removal, using hammers and airlift dredges, of the 2 species (red, *Strongylocentrotus franciscanus*, and purple, *S. purpuratus*) of sea urchins most responsible for damage to kelp resources. These techniques and their effects on managing and restoring giant kelp forest in southern California have been reviewed recently by McPeak and Barilotti (1993) and Dean and Deysher (1994).

A fishery for red sea urchins began in California during the mid-1970s to supply roe ("uni") to markets in Japan (Kato and Schroeter 1985). Only well-fed red sea urchins with good quality roe are harvested. These sea urchins are usually collected along the front or within the forest. Sea urchins in the barrens have poor quality roe, or no roe, and are not harvested. The fishery has reduced overgrazing by red sea urchins in some areas along the coast and islands of California. Also, a major market for the smaller purple sea urchin has developed in the 1990s (Kalvass[4] 1992). Both species of sea urchins continue to destroy giant kelp off Point Loma in southern California. Current restoration programs are designed to encourage commercial sea urchin harvesting that will assist in protecting kelp resources.

If sea urchins become too abundant, destroy the kelp forest, and create urchin barrens, they are of no commercial value. It is important to develop restoration methods that allow for the harvest of sea urchins while preventing the destruction of the forest by them. One of the first documented cases of controlling sea urchin movement was the use of nylon netting as a barrier to contain red sea urchins in Barkley Sound, Vancouver Island, British Columbia, Canada (Pace 1981). The netting encircled a population of red sea urchins and prevented them from grazing nearby macrophytes.

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[3] Clark, M.E. 1969. Dissolved free amino acids in sea water and their contribution to the nutrition of sea urchins. Pages 70-93 *in:* Kelp Habitat Improvement Project, Annual Report, 1968–1969. California Institute of Technology, Pasadena, California, USA.

[4] Kalvass, P. 1992. The northern California commercial sea urchin fishery: A case study. Report NT-CSGCP-028, California Sea Grant, University of California, La Jolla, California, USA.

Studies in northern Chile found that frond whiplash by *Lessonia trabeculata* decreased sea urchin densities on the subtidal, rocky bottom (Vasquez 1992), suggesting a unique approach to protecting giant kelp in southern California. The same effect was observed by Dayton (1975) and Santelices and Ojeda (1984) on intertidal macroalgae. A similar phenomena was observed under a canopy of *Laminaria farlowi*, *Cystoseira osmundacea*, and *Pterigophora californica* off Point Loma, where no sea urchins were found associated with these bottom kelps (McPeak et al.[5] 1974).

The main factors maintaining low sea urchin densities in dense beds of prostrate kelp appears to be plant morphology and water movement. Plants with high flexibility produce a strong whiplash effect over the sea urchin population and cause the sea urchins to leave the area. Therefore, if this effect could be duplicated using "artificial plants", it might be possible to decrease sea urchin densities and protect attached giant kelp. These artificial plants might also act as a barrier to prevent sea urchin re-invasion and subsequent destruction of the kelp forest. The purpose of our study was to determine the effects of artificial plants on 1) the number (density) of 3 sea urchin species in sea-urchin-dominated areas and 2) the survival of adult giant kelp transplanted into sea-urchin-dominated areas. The study evaluated whether artificial plants could be used for protection and restoration of giant kelp forest in southern California.

METHODS

Our study was conducted in the Point Loma kelp forest offshore of San Diego, California (32°42'N, 117°16'W). A large stand of giant kelp, measuring approximately 11 km^2, is located on a broad, gently sloping, pavement-like submerged terrace paralleling the shoreline (Turner et al. 1968). The study area was located along the offshore edge of the kelp bed at a depth of 18 m where high densities of 3 species of sea urchins (red, purple, and *Lytechinus anamesus*) form extensive barren grounds (sensu Lawrence 1975).

Artificial plants were constructed using cement bricks for the holdfast and low-density, 2-mm-thick plastic sheets for the blades (Fig. 1). The bricks weighed 17 kg out of water. Ten blades, each measuring 1.07 m long and 10 cm wide, were attached to a brick. Each blade had 4 holes punched in it and several small pieces of lead (15-g fishing sinkers) attached to help it settle to the bottom. The artificial plants mimicked bottom kelps and were used to reduce sea urchin numbers or function as sea urchin barriers.

Four groups, each of 16 artificial plants, were placed at a depth of 18 m along the edge of the Point Loma kelp bed in a sea-urchin-dominated area. The artificial plants were evenly distributed in a 6 x 6-m area by spacing them 2 m apart. Two contiguous and similar areas, without artificial plants, were surrounded with a nylon line anchored

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[5] McPeak, R.H., D. Bishop, and H.C. Fastenau. 1974. Observations and transplantation studies at Point Loma and La Jolla. Pages 52-71 *in:* Kelp Habitat Improvement Project, Annual Report, 1973–1974. California Institute of Technology, Pasadena, California, USA.

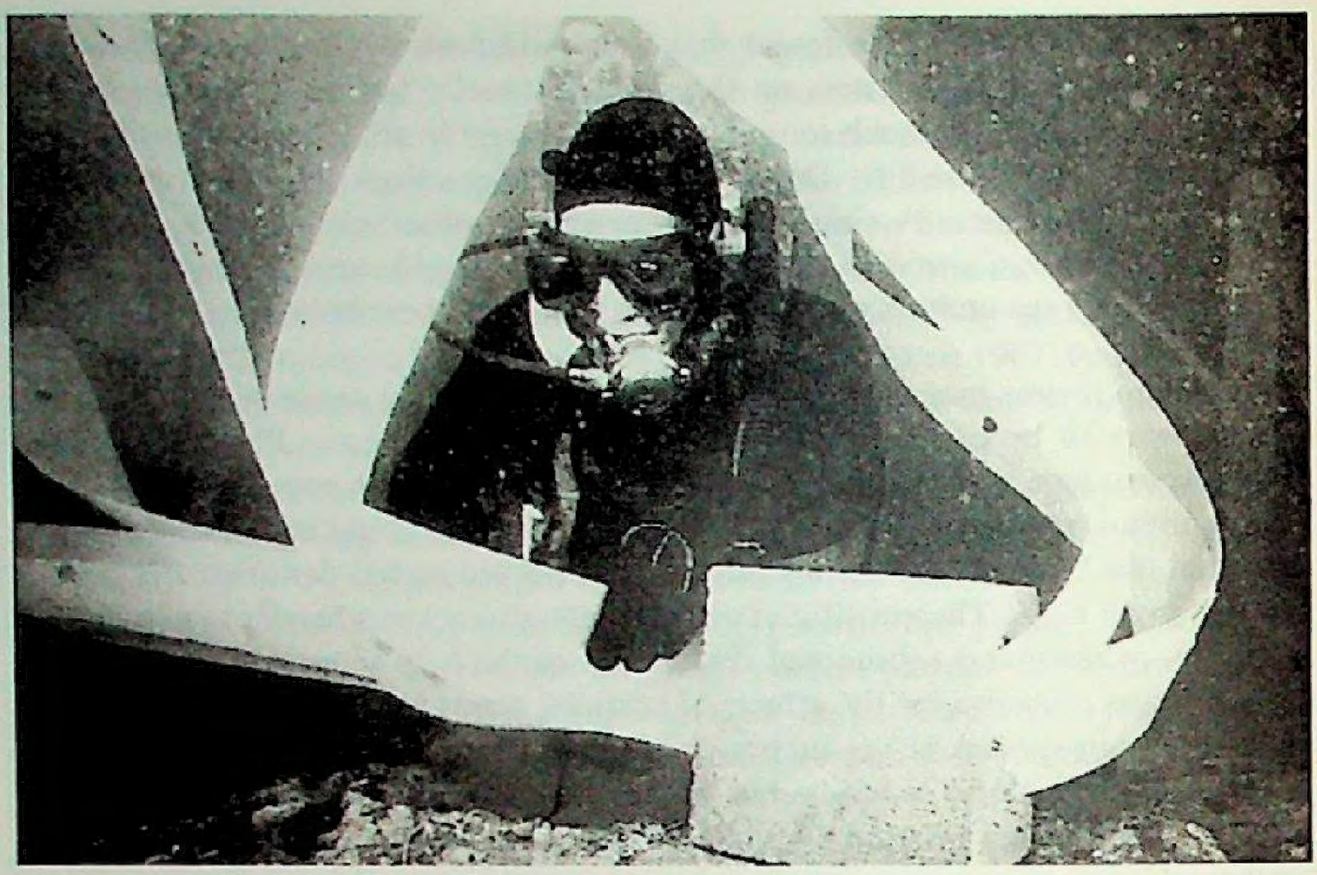

Figure 1. Artificial plants at the Point Loma sea-urchin-dominated area.

at the corners and used as controls. Sea urchin densities were evaluated weekly using a 0.25-m^2 quadrat that was thrown randomly 6 times in each of both experimental and control areas. After 35 days, the approximate natural density of sea urchins was reestablished in the areas containing artificial plants by adding 14 individuals/m^2. Weekly sampling for 10 weeks with the 0.25-m^2 quadrats tested the effect of the artificial plants on the experimental addition of sea urchins.

A 2nd set of experiments was conducted to test the effect of artificial plants on the survival of transplanted giant kelp in sea-urchin-dominated areas. Giant kelp transplants were secured to the bottom using 4 concrete nails, 4 tie-wraps, and 1 rubberband 1.2 cm wide per plant, as described by McPeak and Barilotti (1993). Six groups of 15 adult plants each were collected at a depth of 12 m off Point Loma. These transplants were <1 year old, with holdfast diameters ranging from 18 to 22 cm and averaging 8 fronds each. Two of these 15-plant groups were surrounded by 16 artificial plants each. The artificial plants were evenly distributed around the perimeter of the giant kelp group like a fence. All sea urchins had previously been removed from these experimental areas. As controls, 2 groups of giant kelp plants were transplanted without artificial plants, but with all sea urchins removed (Control 1) and 2 other groups were transplanted without artificial plants and also without sea urchin exclusion (Control 2). The number of stipes, plant survival, and sea urchin density (number/m^2) were measured weekly for 10 weeks for each of the 15 plants in all the experimental areas.

RESULTS

Effects of Artificial Plants on Sea Urchin Densities

The whiplash effect of the artificial plants affected the 3 species of sea urchins differently. Artificial plants decreased red sea urchin average densities significantly ($t = 4.26$, $df = 30$, $P < 0.001$), from 4/m^2 to near 0/m^2, whereas the control remained at 3–4/m^2 ($t = 0.67$, $df = 30$, $P > 0.05$) (Fig. 2A). Purple sea urchin average densities also decreased significantly ($t = 7.36$, $df = 30$, $P < 0.001$), from 15/m^2 to 2/m^2, whereas the control remained at 7–10/m^2 ($t = 0.66$, $df = 30$, $P > 0.05$) (Fig. 2B). More than 70% of the density decrease was observed during the 1st week for both species. *Lytechinus anamesus*, on the other hand, was not affected by the artificial plants and there were no significant differences in areas with ($t = 1.72$, $df = 30$, $P > 0.05$) and without ($t = 1.38$, $df = 30$, $P > 0.05$) artificial plants. *Lytechinus* densities ranged from 7 to 13/m^2 (Fig. 2C). *Lytechinus* is smaller than the other 2 species and

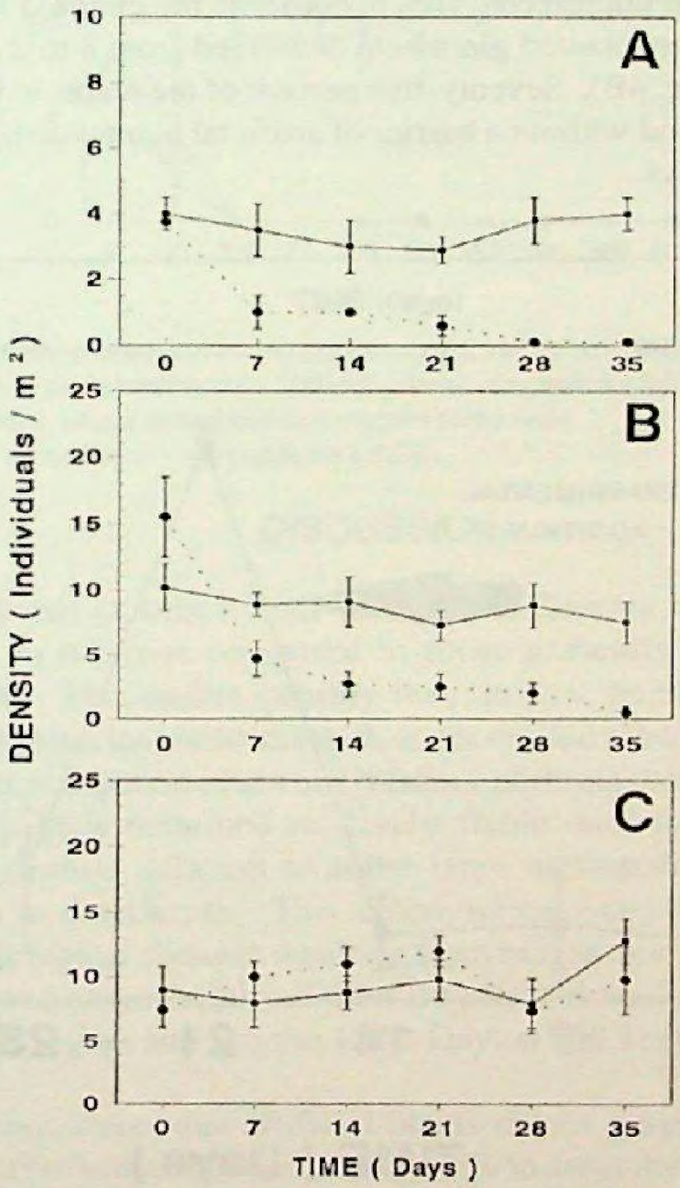

Figure 2. Temporal density variation of (A) red sea urchins, (B) purple sea urchins, and (C) *Lytechinus anamesus* in areas with (- - - -) and without (——) artificial plants. (Mean ± 2SE).

was presumably protected from the whiplash effect of the artificial plants because it was confined to crevices and covered by small cobbles and shell debris.

After 21 days, we experimentally reestablished purple sea urchins to the original density (ca. 14/m^2) in areas where artificial plants were in place. The artificial plants again significantly ($t = 7.63$, $df = 28$, $P < 0.001$) decreased the sea urchin numbers from 14 to 2/m^2 within 2 weeks (Fig. 3).

Effects of Artificial Plants in Transplanting Experiments

All transplanted adult giant kelp survived in the experimental areas protected by artificial plants during the 70 days of the study period (Fig. 4A). The protected transplants grew during the study and the mean number of stipes per plant increased significantly, from 8 to 14 ($t = 5.46$, $df = 28$, $P < 0.001$) (Fig. 4B). Plants in Control 1, with sea urchins removed but without artificial plants, were grazed. Twenty-five percent of the plants died in 7 days and all were dead in 14 days because sea urchins quickly moved into the unprotected area to consume the plants (Fig. 4A). The mean number of stipes of transplanted giant kelp decreased from 8 to 2 in the first 7 days, and to 0 in 14 days (Fig. 4B). Seventy-five percent of the plants in Control 2 (without sea urchins removed and without a barrier of artificial plants) died within 7 days and all were dead in 14 days.

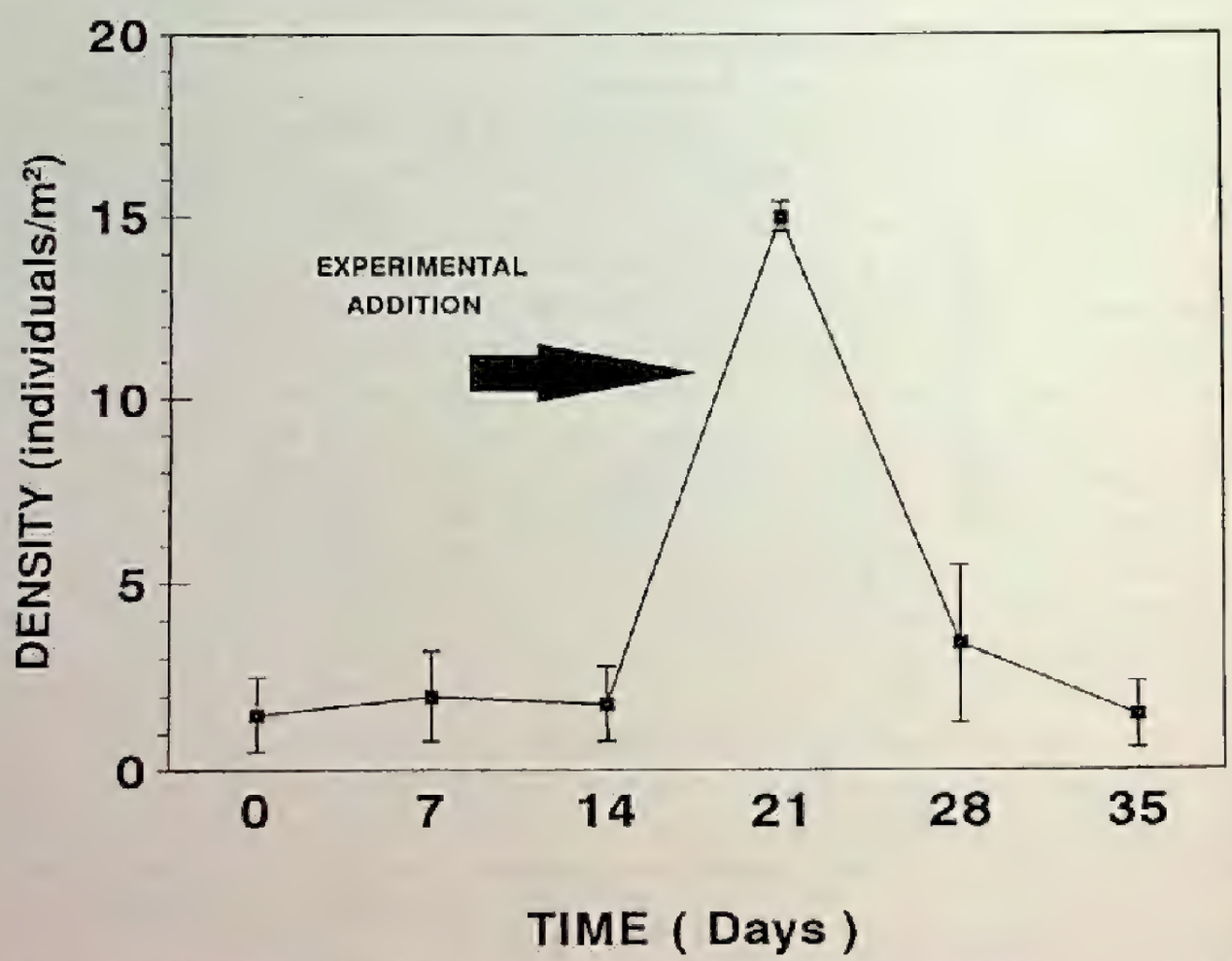

Figure 3. Temporal density response to the experimental addition of purple sea urchins in an area with artificial plants. (Mean ± 2SE).

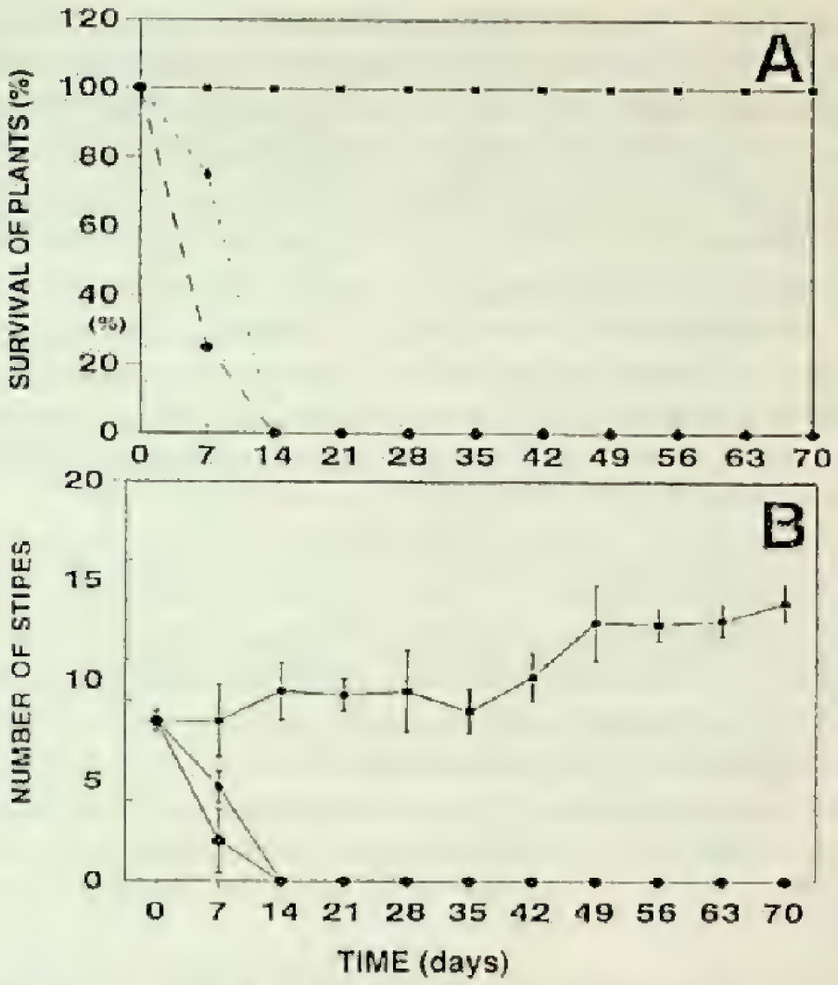

Figure 4. Giant kelp transplant survival (A) and temporal variation in the number of stipes per giant kelp transplant (B) in areas protected by artificial plants. Experimental area protected by artificial plants (———); Control 1, unprotected and sea urchins removed (.....); and Control 2, unprotected and sea urchins not removed (- - - - -). (Mean ± 2SE).

DISCUSSION

Long-term records of kelp forests in central California suggest that they were relatively stable in the past compared to those presently occurring near large metropolitan areas. This earlier stability may, in part, be attributable to sea otter foraging on herbivorous invertebrates such as sea urchins (Estes and Steinberg 1988). Following the near extirpation of sea otters from California in the early 1800s, records indicate that kelp beds remained relatively stable until the mid-1900s. In the mid-1940s, kelp forests adjacent to some large metropolitan areas in southern California began to deteriorate. This deterioration was attributed to a complex combination of biological changes resulting from human activity (e.g., domestic and industrial wastes and commercial and sport fisheries) as well as natural factors, such as El Niño events (Tegner and Dayton 1981, Dayton and Tegner 1990, Dayton et al. 1992).

Our study demonstrates that artificial plants reduce purple sea urchin densities by nearly 85% and red sea urchin densities by 75% in areas dominated by sea urchins. Movement of the blades and the whiplash effect of the artificial plants "herded" the sea urchins out of the area. Even though sea urchin densities were lowered by artificial

plants, we do not believe that giant kelp would develop in these areas from spores or microscopic stages if the artificial plants were left in place because the whiplash effect would also continually remove the young plants. The whiplash effect of a natural population of *Laminaria farlowi* was studied off Point Loma in areas where *L. farlowi* was present (control area) and where it had been removed (McPeak et al.[5] 1974). Giant kelp developed within 1 month in areas where *L. farlowi* was removed, but never developed in control areas. Presumably, microscopic gametophytes and sporophytes of giant kelp were present, but the whiplash effect and shading did not allow giant kelp to develop until *L. farlowi* was removed. As with *L. farlowi*, it would be necessary to remove artificial plants before giant kelp would develop. Thus, it may be more economical to simply remove the sea urchins using hammers or airlift dredges.

Artificial plants have the greatest potential for use in restoration when they are used to protect natural populations of giant kelp from a moving front of sea urchins and to protect transplanted giant kelp from benthic grazers. Red and purple sea urchins have been observed destroying giant kelp beds at the rate of 10 m per month (Leighton 1971). Our study demonstrates that artificial plants effectively prevent sea urchins from destroying giant kelp transplants placed in sea-urchin-dominated areas. Sea urchins were absent or present at low densities in areas protected by the artificial plants. Establishing a barrier of artificial plants between a sea urchin front and a giant kelp forest can prevent destruction of the forest by the grazing sea urchins.

Although our study indicates that artificial plants can also protect transplanted giant kelp in sea-urchin-dominated areas, a better design for artificial plants might be even more effective. This involves constructing a long row of plants attached to a nylon line. The line, with attached plastic blades, could be secured to the rocky substrate using fasteners. The string of plants could be quickly installed in the appropriate area to provide maximal protection of giant kelp.

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DEER GENDER DETERMINATION BY POLYMERASE CHAIN REACTION: VALIDATION STUDY AND APPLICATION TO TISSUES, BLOODSTAINS, AND HAIR FORENSIC SAMPLES FROM CALIFORNIA

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We determined the gender of deer samples using polymerase chain reaction primers to the *sry* gene located on the Y chromosome of mammalian males. Primers to the ZFX/ZFY genes were added to the same amplification reaction as a control against amplification failure. The *sry* fragment amplified from deer DNA was partially sequenced. Sequence alignment with published bp sequences from other mammals and gel migration indicate the fragment is 174 bp long. In a blind test, tissue samples from 100 individual deer and elk of known sex were correctly gender-typed. DNA from deer bloodstains and hair samples was successfully amplified and typed. Under known limiting DNA conditions, the duplex reaction results were reliable and no false positive females were observed. DNA detection on gels stained with SYBR green was more sensitive than with ethidium bromide. DNA from wildlife forensic samples confiscated between 1990 and 1997 was extracted with Chelex 100 and tested. Twenty-four out of 31 different evidence items tested were successfully gender-typed: 15 out of 21 bloodstains, 5 out of 6 hairs, and 4 out of 4 tissue samples.

INTRODUCTION

The mule deer, *Odocoileus hemionus*, is the most abundant big game mammal of western North America (Leopold et al. 1981). Sex specific take of deer governs most hunting in California. Hunting of females is prohibited in most parts of the state. After an illegally harvested female deer has been butchered, the perpetrator traditionally has been safe from apprehension because primary sex characteristics have been removed. Due to the nature of deer and wildlife poaching, bloodstains and hairs are very common pieces of wildlife evidence. The ability to gender-type a small piece of tissue, a bloodstain, or hair with a simple, robust, accurate method in the forensic laboratory allows prosecutors to more effectively enforce game laws.

DNA analysis is a powerful and widespread tool in wildlife studies, with broad applications in management, conservation, and forensics (Cronin et al. 1991, Blackett

and Keim 1992, Guglich et al. 1993, Kohn et al. 1995, Murray et al. 1995, Travis and Keim 1995). One such application is the sex determination of biological samples by the polymerase chain reaction (PCR), based on differential amplification of nucleotide sequences from the X and Y chromosomes in female and male mammals (Aasen and Medrano 1990, Amstrup et al. 1993, Griffiths and Tiwari 1993, Taberlet et al. 1993, Schwerin and Pitra 1994, Fain and Lemay[1] 1995, Wasser et al. 1997).

We determined the gender of deer samples by PCR with a set of primers for the dual amplification of fragments from the X and Y chromosomes. We used primers designed by Fain and Lemay[1] (1995) for the amplification of a fragment from the *sry* gene located on the Y chromosome of male mammals. Females lack a Y chromosome and the test is based on the absence of an sry PCR product in females. As a control against amplification failure, the primers for the homologous *ZFX/ZFY* genes on the X chromosome of females (ZFX) and XY chromosomes of males (ZFX/ZFY) (Aasen and Medrano 1990, Wasser et al. 1997) were included in the same reaction to demonstrate amplification success. Both pairs have been shown to work in several mammalian groups (Aasen and Medrano 1990, Fain and Lemay[1] 1995), but the mule deer was not included among those. The objectives of our study were to test and validate the primers for gender identification of the mule deer and black-tailed deer, *O. h. columbianus*, complex and the elk, *Cervus elaphus*, and evaluate the procedures with forensic samples from adjudicated wildlife casework.

METHODS

The California Department of Fish and Game (CDFG) Wildlife Forensic Laboratory (WFL) provided the samples for the laboratory studies at the University of California School of Medicine (UCD). The deer and elk samples for the validation study came from road-killed animals and hunter check stations throughout California. The forensic samples were from confiscated material collected by CDFG game wardens from 1990 to 1997.

Highly purified genomic DNA for use as the standard was prepared from 10 g of deer muscle tissue by a phenol and chloroform procedure. This standard organic extraction protocol was received from the National Wildlife Forensics Laboratory of the Fish and Wildlife Service in Ashland, Oregon. DNA prepared by this method was used to optimize the PCR conditions described below in the amplification section. Eight of these samples were also used in a blind trial, in which the gender was not known to the person performing the identifications. The total numbers of muscle and organ samples ultimately included in the blind trials to validate the gender typing procedure were 78 mule deer and 22 elk.

Ninety-two of the 100 samples had their DNA extracted by a rapid Chelex 100 (Chelex 100, Bio-Rad Laboratories[2], Richmond, California, USA) procedure using

[1] Fain, S.R. and P.J. Lemay. 1995. Gender identification of humans and mammalian wildlife species from PCR amplified sex-linked genes. Abstract in Proceedings of the American Academy of Forensic Science Annual Meeting, Seattle, Washington, USA.

[2] Reference to trade names does not imply endorsement by the California Department of Fish and Game.

muscle or organ tissue, as follows: 25–50 mg of tissue was added to 400 µl of 5% Chelex 100 (weight/volume) and 25 µl of Proteinase K (from a 20 mg/ml stock solution) and incubated for 2 hours at 65°C with the samples rotating gently. Each sample was then vortexed and centrifuged for 3 minutes and the supernatant discarded, leaving the pellet covered by a small amount of supernatant. The pellet was resuspended in 200 µl of 5% Chelex 100, mixed, then boiled for 10 minutes with the tubes fitted with Sherlock cap locks (Sherlock Micro-centrifuge Cap Locks, USA Scientific Plastics[2], Ocala, Florida, USA). The samples were vortexed for 10 seconds and centrifuged for 2 minutes. One hundred µl of each supernatant was transferred to a fresh tube and stored at -20°C. One extraction blank tube containing all the reagents, but no biological samples, was processed with each set of extractions. This blank extraction control was run in the PCR with the extracted samples.

Bloodstains were prepared on slides with whole blood from deer of known gender and subjected to treatments designed to mimic wildlife forensic conditions (Table 1). Slides were frozen immediately (samples 1 and 13), left in the incubator at 37°C for several days (samples 2, 21, and 22) or left in direct sunlight for several hours (samples 3 and 16).

Hairs from 1 male and 1 female deer were collected with sterile gloves, placed in separate plastic bags, and kept at room temperature until processing. The hairs were processed blind, i.e., the gender of origin was unknown to the person performing the test. Individual hairs were examined under the microscope and cut into 2 sections, 1 short (0.5 cm), containing the root bulb, and 1 with the shaft only. Each section was labeled on the basis of the pigmentation: white, brown, or black.

Thirty-one forensic samples from 22 adjudicated wildlife cases were processed. The evidence had been confiscated by CDFG game wardens from 1990 to 1997 and consisted of 21 bloodstains, 6 hairs, and 4 tissues (Table 2). The substrates from which they were recovered varied from hard surfaces such as knife metal to different types of cloth.

DNA from blood and hair was extracted with Chelex 100 following previously described protocols (Taberlet et al. 1993, Walsh et al. 1991). The oven incubation time for hairs was changed to 2 hours, except where noted.

Two sets of oligonucleotide primers were used simultaneously in each amplification reaction. One set of primers amplifies a 442–445 bp fragment from the *ZFX/ZFY* genes in males and females (Aasen and Medrano 1990). The other

Table 1. Treatments of deer bloodstains on slides.

Sample #	Exposure
1	Frozen immediately
2	4 days in incubator at 37°C
3	1 hour 10 minutes in direct sunlight
4	5 hours in direct sunlight
13	Frozen immediately
16	3 hours 30 minutes in direct sunlight
21	11 days in incubator at 37°C
22	13 days in incubator at 37°C

set of primers amplifies a fragment 224 bp long from the *sry* gene in males only. Because the test with the *sry* primers is based on the absence of the 224-bp product in females, the ZFX/ZFY primers act as internal positive control to show that the lack of a 224-bp fragment is not due to an amplification failure and demonstrate the presence of amplifiable DNA in the PCR reaction. Male DNA samples show 2 PCR products (445 bp and 224 bp), whereas female samples show only 1 PCR product (224 bp). The *sry* primer sequences (Fain and LeMay[1] 1995) were as follows:

Y53-3C 5'-CCC ATG AAC GCA TTC ATT GTG TGG-3'

Y53-3D 5'-ATT TTA GCC TTC CGA CGA GGT CGA TA-3'

The primers were synthesized at the Protein Structure Laboratory of the University of California at Davis. The optimized PCR conditions were: 95°C for 1 minute,

Table 2. Biological materials submitted for analysis, substrates from which they were recovered, and gender determination results of adjudicated case work samples from deer and elk.

Case number	Date	Material	Subtrate	Species	Gender
4	August 1992	Bloodstain	Metal (knife)	Deer	Female
7	August 1993	Bloodstain	Metal (knife)	Deer	Female
19	August 1996	Bloodstain	Metal (knife)	Deer	Female
19	August 1996	Hair	Metal (gambrel)	Deer	Female
6	October 1992	Bloodstain	Metal (knife)	Deer	Male
8	October 1993	Bloodstain	Metal (knife)	Deer	Male
8	October 1993	Bloodstain	Metal (machete)	Deer	Male
8	October 1993	Bloodstain	Metal (knife)	Deer	Male
17	November 1995	Bloodstain	Metal (rifle bolt)	Deer	Male
20	October 1996	Bloodstain	Metal (knife)		Mixed genders
1	February 1990	Bloodstain	Metal (saw)		No product
11	July 1994	Bloodstain	Cloth (sleeping bag)	Deer	Female
24	March 1997	Bloodstain	Cloth (unspecified)	Deer	Female
20	October 1996	Bloodstain	Cloth (sheet)	Deer	Male
5	August 1992	Bloodstain	Cloth (jeans)		No product
9	October 1993	Bloodstain	Cloth (shirt)		No product
10	October 1993	Bloodstain	Paper		No product
21	September 1996	Bloodstain	Paper		No product
15	October 1995	Bloodstain	Weatherstrip		No product
17	November 1995	Bloodstain	Unspecified	Deer	Female
14	November 1995	Bloodstain	Unspecified	Deer	Male
21	September 1996	Bloodstain	Rock	Deer	Male
21	September 1996	Hair	Rock	Deer	Male
18	February 1996	Frozen meat	Frozen meat	Deer	Female
12	September 1995	Frozen meat	Frozen meat	Deer	Male
23	December 1996	Frozen meat	Frozen meat	Deer	Male
22	November 1996	Dried meat	Dried meat		Male
2	January 1992	Hair	Hair	elk	Male
10	October 1993	Hair	Hair	Deer	Male
16	November 1995	Hair	Hair	Deer	Male
17	November 1995	Hair	Hair		No product

35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 73°C for 60 seconds. All reactions were conducted using an "EasyCycler" thermal cycler (EriComp, Inc.[2], San Diego, California, USA). A total volume of 30 µl per PCR tube was used. The 30 µl was composed of 25 µl of the master reaction mix already containing the buffer to load the PCR products directly onto the gel (Appendix A), 3 µl of Taq polymerase dilution containing 1.5 units of Taq DNA polymerase, and 2 µl of the DNA extract from the tissue to be amplified. The master mix in Appendix A is for 10 amplification reactions. The surface of each reaction mix was covered with 40 µl of mineral oil. The amplification conditions for tissues, blood, and hair were the same except for the DNA extract volume added to the reaction mix: 2 µl of tissue DNA extract and 9 µl of blood or hair DNA extract were added. To accommodate different DNA extract volumes and still retain the proper molar concentrations in the reaction mix, the volume of water added to the reaction mix was reduced when 9 µl of extract was used (Appendix A). Positive controls consisting of known male and female DNA samples were run with each PCR, as well as 2 negative controls consisting of an extraction reagent control blank and a PCR reagent control blank. PCR blank refers to a reaction containing all the reagents, but no DNA; 2 µl of water were added to it.

The DNA concentration in the highly purified standard sample (the phenol/chloroform extract) was determined with a spectrophotometer (Lambda 3A, Perkin Elmer Corp.[2], Norwalk, Connecticut, USA). A serial dilution of this extract was used in a sensitivity test to determine the primer's amplification limits. DNA from the standard sample varying from 12 ng to 1.2 pg in amount was added to the PCR reaction in duplicate. All PCR products were electrophoresed on 1.2% agarose mini-gels and evaluated by UV photography. Ten µl of the PCR product, already containing the dye Cresol red in sucrose as gel loading buffer (Appendix A), were added to each well. The gels were run for 45–60 minutes at 90 V. The DNA was stained by immersing the gels for 4 minutes in ethidium bromide (10 µg/ml), followed by destaining in water for 30 minutes. Duplicate gels were stained with SYBR Green I, as per the manufacturer's recommendations (FMC Bioproducts, Molecular Probes, Inc.[2], Rockland, Maine, USA).

The general procedures followed the recommendations from the Federal Bureau of Investigation[3] (1993) manual for Dqα Typing Protocol. In brief, different laboratory rooms were used for pre-PCR procedures, such as sample preparations, DNA extractions, and reagent preparation and for the post-PCR procedures, such as running the gels. Dedicated sets of micropipettors were reserved in those rooms and all reagent and sample transfers were done with aerosol-resistant tips.

RESULTS

The fragment generated by the primers to the *sry* gene was confirmed by sequencing some of the amplified fragment from a black-tailed deer from Sacramento

[3] Federal Bureau of Investigation Laboratory. 1993. Dqα typing protocol. August 4, 1993. Washington, D.C., USA.

County. The sequencing was performed by the single strand, single pass technique, in which both strands were sequenced (Lark Sequencing Technologies, Houston, Texas) (Table 3). The fragment was not sequenced in its totality, but alignment with published *sry* sequence from cattle (Payen and Cotinot 1993) indicates that it is 174 bp long. The total length of the PCR product, with the *sry* primers (50 bp), is 224 bp. Fig. 1 (lane 6) shows the 2 PCR products amplified from a male deer genomic DNA: 224 bp and 445 bp. Lane 7 shows 1 band only (445 bp) amplified from a female deer sample.

The results of the primers' amplification limits show that a minimum of 120 pg of starting DNA is necessary for gender identification with both sets of primers in the same reaction tube when the PCR is conducted as noted above. Twelve pg of starting DNA produced a weak band on the gel, but the bands are barely detectable (Fig. 2). DNA detection was more sensitive when gels were stained with SYBR Green I than with ethidium bromide (data not shown). SYBR Green I was retained as the standard staining procedure.

DNA amplification from 8 deer blood smears kept several days at 37°C or exposed to sunlight was successful and the 4 male and 4 female samples were correctly identified (Table 1). The gender of origin from single deer hair (3/3) was determined when the hair root, or part of it, was present, but not from hair shaft alone. One of the extracts from a hair with black pigmentation did not amplify initially. A PCR reaction containing control DNA and spiked with the hair extract was inhibited. This hair extract was successfully amplified after purification with a standard alcohol precipitation procedure using isopropanol, as described in Hanni et al. (1995).

In the blind trial with 100 tissue samples, the gender of 78 individual deer and 22 elk was correctly identified. Ninety-two of these 100 samples had been extracted with Chelex 100 and some of these extracts initially failed to amplify. These tissue samples were re-extracted with 1 resuspension step added to the extraction protocol. All these samples were then successfully amplified and sexed. The resuspension in 200 µl of 5% Chelex 100 was retained in the final extraction protocol reported in Methods.

In the survey of adjudicated cases, the gender was determined for 24 samples out of 31 attempted (Table 2). These samples came from 22 different cases. DNA from

Table 3. *Sry* nucleotide sequence from black-tailed deer.

```
          10                  20                  30                  40
GGTGG CTCTA GAGAA TCCCA AAATG CAAAA CTCAG AGATC AGCAA

    50                  60                  70                  80                  90
5'GCAGC TGGGG TATGA GTGGA AAAGG CTTAC AGATG CTGAA AAGCG

         100                 110                 120                 130
CCCAT TCTTT GAGGA GGCAC AGAGA CTACT AGCCA TACAC AGAGA

   140                 150                 160
CAAAT ACCCG GGCTA TA 3'
```

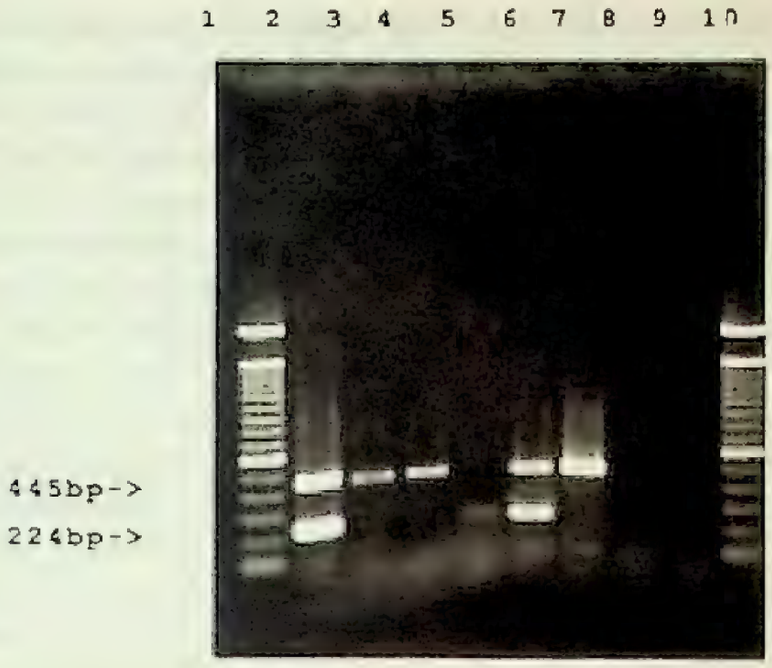

Figure 1. Amplification results of bloodstain and hair samples from adjudicated cases (unknown gender of origin). Lane 2–3: DNA extracted from bloodstains on rifle bolt and knife respectively. Lane 4–5. DNA extracted from 1 deer and 1 elk hair, respectively. Lanes 1 and 10: molecular ladder marker (100 bp, Gibco, Life Technologies[2], Rockville, Maryland, USA). Lane 6: positive PCR control (DNA from a known male deer). Lane 7: positive PCR control (DNA from a known female deer). Lane 8: extraction blank. Lane 9: negative PCR control (water added, no DNA).

Figure 2. Sensitivity results of the simultaneous amplification of X- (445 bp) and Y- (224 bp) specific fragments following the serial dilution of starting DNA added to the PCR. Lanes 1–5: 12.6 ng, 1.26 ng, 0.12 ng, 0.012 ng, 0.0012 ng of starting DNA. Lane 6: molecular ladder marker (100 bp, Gibco, Life Technologies[2], Rockville, Maryland, USA). Lane 7: positive PCR control (DNA from known male deer). Lane 8: negative PCR control (water added, no DNA). Lanes 9–10, empty. 1.2% agarose gel (Ultrapure, Gibco[2]). DNA visualized under UV light after gel-staining. SYBR-stained gel.

bloodstains was amplified in 15 out of 21 samples. Nine out of 10 bloodstains recovered from metal substrates were amplified and gender-typed. The negative sample contained inhibitors to the PCR, even after serial dilutions and purification with S-400 microspin (S-400 microspin columns, Pharmacia BioTech[2], Baie D'urfe, Quebec, Canada) columns. Three out of 5 bloodstains recovered from cloth were gender-typed. The 2 negative samples contained no inhibitors but still failed to amplify after ethanol precipitation and concentration. Gender identification was also successful with 5 out of 6 hairs examined. One of the hairs tested came from a gambrel seized by CDFG wardens at the same time as a bloodstained knife (Table 2, case 19). Both the hair and the bloodstain on the knife were found to be from a female. One hair from an elk yielded an amplification product only after overnight chelex incubation. Four tissue samples were processed: 3 frozen and 1 dried sample kept at room temperature for an unknown period of time. This sample required an overnight oven incubation to yield sufficient DNA to produce an amplification product in the PCR. The 4 tissue samples that were gender-typed showed 1 out of the 4 to have originated from a female (Table 2, case 18).

DISCUSSION

Two major concerns in forensic procedures are the reliability and sensitivity of the techniques. The reliability of the techniques reported here was tested by 1) using the *sry* primers to determine the gender of California deer and elk, 2) using 2 sets of primers to eliminate the possibility of misidentifying a male as a female, and 3) routine handling of large numbers of samples without contamination. Although the exquisite sensitivity of the PCR makes it a useful forensic test, the sensitivity also creates a potential for cross-contamination of samples. The blind test results validated the use of the *sry* primers with California deer and elk. The reliability of the technique was also tested when applied to simulated and actual forensic samples.

These results also showed that the handling procedures were reliable, because no female was misidentified as a male and no contamination was observed either in the extraction blanks or in the PCR negative controls. The number of transfers while handling forensic samples is very important. The protocol for tissue extraction requires only 1 transfer of the sample from 1 tube to another before storage. This transfer step was added at the end of the extraction protocol when it was observed that some tissue extracts had a decreasing amount of DNA after repeated freezing and thawing.

The reliability of the technique in relation to its sensitivity was 100%. In the sensitivity test, either both the *sry* and ZFX/ZFY fragments were amplified or no product was detected with decreasing amount of high purity DNA from a male deer added to the PCR reaction (Fig. 2). With degraded DNA from forensic samples, the DNA yield for the fragment generated by the *sry* primers (224 bp) was sometimes greater than for the internal control (445 bp), as evaluated by UV photography (Fig. 1). The reaction appeared to favor the amplification of the shorter fragment, i.e., the *sry* fragment; hence, false female positive tests never resulted from male DNA. This further supports the reliability of this system with 2 sets of primers.

The detection limit of this system was 120 pg of starting DNA. This limit is well within the reports for primers used in human forensic investigations. Kreike and Lehner (1995) reported 50 pg as the limit with both sets of primers being tested separately. Of various approaches based on DNA amplification for human gender tests, a recent one favored the use of new *ZFY/ZFX* primers, followed by restriction enzyme digestion (Reynolds and Varlaro 1996). That PCR required 500 pg of starting DNA to yield enough product for the digestion step. This approach was based on the possibility of misidentifying a male for a female if there was a failure of amplification of the *sry* fragment without an amplification failure of the internal control because each primer set is run in a different reaction. We never observed an amplification failure of the *sry* fragment without an amplification failure of the *ZFX/ZFY* internal control with the 2 sets of primers reported in this study. The size of the *ZFX/ZFY* internal control fragment (445 bp) does not appear to pose a problem. Our findings were reproducible with decomposed meat samples, aged blood subjected to various known conditions of degradation, and hair kept several months at room temperature. Some samples may fail to produce an amplification product, as with the forensic samples, but, of the 100 validation tissue samples, blood smears, and hairs tested, we never misidentified the gender of origin.

While not the topic of this study, the primers tested here for deer gender identification were reported to work with other mammal species (Fain and LeMay[1] 1995). In our tests, DNA from human male and female blood and single hairs was successfully amplified and gender-typed (data not shown).

As a routine procedure for detection, SYBR Green I was superior to ethidium bromide (Schneeberger et al. 1995) and was adopted as our standard staining procedure because it is more sensitive and as easy to use as ethidium bromide.

The procedures described here are simple, robust, and fast. From start to finish, only 3 sample transfers are required: 1 to store the extract, 1 to add to the PCR reaction, and 1 to load the PCR product onto the gel. Results can be obtained in 1 day.

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Appendix A. Master mix preparation for 10 reactions.

	Volume (µl) reagent for 2 µl extract	Volume (µl) for 9 µl extract
A. Double distilled water, autoclaved	122.5	52.5
B. 10X thermophilic buffer with 15mM $MgCl_2$ (Promega)	30.0	30.0
C. $MgCl_2$ (25mM)	12.0	12.0
D. Dinucleotides (10 mM each)	6.0 µl of each 24 µl total	6.0 µl of each 24 µl total
E. Primers 20 pmoles/µl		
1	3.0	3.0
2	3.0	3.0
3	3.0	3.0
4	3.0	3.0
F. Bovine serum albumin 10 mg/ml	4.5	4.5
G. Cresol Red 1 mM[a]	30.0	30.0
H. Sucrose 40%[a]	15.0	15.0
	250.0	180.0
Each PCR tube contained	25 µl reaction mix 2 µl extract from sample 3 µl *Taq* 30.0 µl	18 µl reaction mix 9 µl extract from sample 3 µl *Taq* 30.0 µl

[a] Gel loading buffer

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VALIDATION OF DAILY INCREMENTS IN OTOLITHS OF NORTHERN SQUAWFISH LARVAE

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Otoliths from laboratory-reared northern squawfish, *Ptychocheilus oregonensis,* larvae were examined to determine the periodicity of increment deposition. Increment deposition began in both sagittae and lapilli after hatching. Reader counts indicated that increment formation was daily in sagittae of 1–29-day-old larvae. However, increment counts from lapilli were significantly less than the known ages of northern squawfish larvae, possibly because some increments were not detectable. Otolith readability and age agreement among readers were greatest for young (<11 days) northern squawfish larvae. This was primarily because a transitional zone of low-contrast material began forming in otoliths of 8–11-day-old larvae and persisted until approximately 20 days after hatching. Formation of the transition zone appeared to coincide with the onset of exogenous feeding and continued through yolk sac absorption. Our results indicate that aging wild-caught northern squawfish larvae using daily otolith increment counts is possible.

INTRODUCTION

Studies of otolith microstructure have been used to provide important information on early life history parameters for a number of fish species since the verification of daily increments in otoliths of some temperate and tropical fishes (Brothers et al. 1976). The presence of daily increments in otoliths enables researchers to determine hatching dates and ages of individual larval and juvenile fish and thereby estimate parameters such as timing of spawning and age-specific growth and mortality rates (Miller and Storck 1984, Essig and Cole 1986, Graham and Orth 1987, Isely and Noble 1987). Daily increments in otoliths are not always present, however, and the age at first increment deposition is not consistent among all taxa. For this reason, studies validating daily increment deposition should be conducted before estimating ages with otoliths (Campana and Neilson 1985).

We conducted an otolith increment validation study on larvae of the northern squawfish, *Ptychocheilus oregonensis*, a large cyprinid native to the Columbia River basin. Management of the northern squawfish is an important issue in the Pacific Northwest because it is a major predator on outmigrating juvenile anadromous salmonids (Reiman et al. 1991). As part of a larger research effort directed at gaining a better understanding of the early life history of northern squawfish in the

Columbia River Basin, it was necessary to estimate ages of larvae through otolith analysis. In the current study, otoliths of known-age, laboratory-raised northern squawfish larvae were examined to determine age at first increment formation and if increments were deposited daily.

METHODS

Laboratory Culture and Sampling

Adult northern squawfish collected from the lower Columbia River were injected with carp pituitary extract and hand-spawned. Fertilized eggs were placed in containers with flow-through water systems and incubated at 15–16°C. The eggs hatched over a period of 1–2 days after 8–10 days of incubation and began feeding 8 days post-hatching. Larvae were fed frozen brine shrimp, *Artemia* spp., once or twice daily and held at ambient photoperiods. Post-hatch daily water temperature in the holding containers averaged 15.6°C (SD = 0.2, n = 33) for the duration of the experiment.

Northern squawfish larvae (n = 10) sampled during hatching were assigned a known age of 0. Thereafter, larvae (n = 4–8) were generally sampled on alternate days until 33 days after hatching. Sampled larvae were anesthetized with MS-222, measured to the nearest 0.1 mm standard length using a dissecting microscope fitted with an ocular micrometer, and preserved in 95% ethanol.

Processing and Increment Counts

Sagittae and lapilli were teased free with a fine probe under a dissecting microscope. Maximum diameters of sagittae and lapilli from recently hatched larvae (n = 12) were measured using a compound microscope fitted with a calibrated ocular micrometer at 400X magnification. Otoliths from early (1–9 days old) larvae were mounted concave-side up on microscope slides in a clear, low-viscosity acrylic medium (Cytoseal, Stephens Scientific[1], Riverdale, New Jersey, USA) and were not processed further. Otoliths from older (10–33 days old) larvae were mounted concave-side up on microscope slides in thermoplastic cement and polished against a wet felt pad with 0.3 µm alumina suspension until a level plane from the core to the posterior edge was obtained. After heating the slides to liquefy the thermoplastic cement, the otoliths were flipped and polished on the convex surface until all inner increments appeared to be viewable (Miller and Storck 1982).

We followed the protocol of Miller and Storck (1982) for counting increments. Subdaily increments were occasionally present, but were readily distinguished from daily increments because they were narrower and fainter. Processed otoliths were read in random order. Three readers counted the number of increments in each otolith 3 times. Averages of the 3 counts obtained by each reader were then compared. If the average counts differed among readers by <10% or <4 increments, they were

[1] The use of trade names does not imply endorsement by the U.S. Geological Survey or the California Department of Fish and Game.

accepted and averaged (Miller and Storck 1982). When disagreement occurred, otoliths were set aside and increments were recounted a 2nd time. If disagreement occurred a 2nd time, the otoliths were excluded from analysis (Parrish et al. 1994). To determine if increment counts provided accurate estimates of known age, average increment counts were regressed on the known ages of larvae in days. A 1:1 relationship, and correct aging, was assumed if the slope of the line did not differ significantly (P <0.05) from 1.0.

RESULTS

Sagittae and lapilli of recently hatched larvae (<2 days old) were circular and had mean diameters of 55.3 µm (SD = 5.2, n = 12) and 52.0 µm (SD = 5.1, n = 9), respectively. Asterisci, the smallest of the 3 otolith pairs, generally formed 10–15 days after hatching. The larvae ranged in size from 6.5–7.1 mm (n = 4) at hatching to 11.5–11.8 mm (n = 4) at 33 days.

Regressions of increment counts on the known ages of larvae demonstrated that sagittal increment counts accurately depicted the true ages of larvae: sagittal increments = 0.54 + 1.00 age in days (r^2 = 0.98, n = 60) (Fig. 1). The slope of the line was not significantly different from 1.0 (F = 0.01; df = 1, 58; P = 0.91).

In contrast to sagittae, mean increment counts for lapilli underestimated the true ages: lapillar increments = 0.53 + 0.87 age in days; r^2 = 0.98, n = 48). The slope of the line was significantly different from 1.0 (F = 48.6; df = 1, 46; P = 0.0001. Intercepts of both regression lines were significantly different (P < 0.05) from 0.

Between-reader increment count variation caused the rejection of a high percentage of otoliths (46.1% of 89 lapilli, 48.7% of 117 sagittae) from the analysis. Daily increments were often weakly expressed and particularly difficult to count in

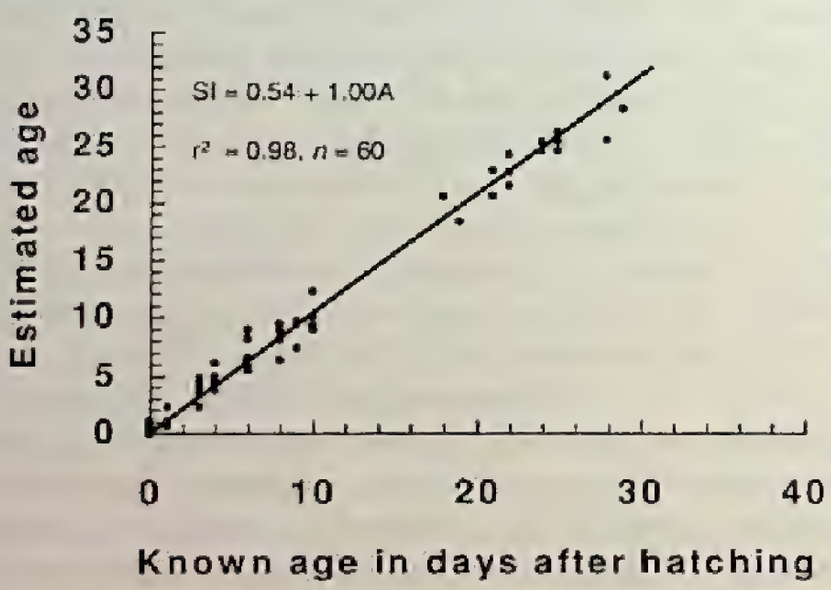

Figure 1. Regression of sagittal otolith increment counts (estimated age) on known ages of laboratory-reared northern squawfish, *Ptychocheilus oregonensis*, larvae. SI = sagittal increment count and A = known age in days after hatching.

both sagittae and lapilli of older (>9–10 days old) laboratory-reared northern squawfish larvae. This led to rejection of a large proportion of age estimates from older fish. Otoliths from fish >9–10 days old were characterized by a transition zone (Kalish 1995) with extremely low contrast increments which made increment counting very difficult. For example, 83% of 54 sagittae from fish <11 days old were accepted for analysis compared to 24% of 63 sagittae from fish >10 days old. The otolith transition zone generally began 8–10 days after hatching and normally persisted for 7–10 days, after which daily increments again became more clearly defined and countable.

DISCUSSION

We validated daily increment formation in sagittal otoliths of laboratory-raised northern squawfish larvae for ages 1–29 days post-hatching. Increment counts from lapilli, however, underestimated the actual ages of northern squawfish larvae. Little information exists on otolith validation in cyprinids to compare with ours. However, Parrish et al. (1994) also found that age estimates from lapilli were lower than those obtained from the sagittae of larval and juvenile walleye, *Stizostedion vitreum*, although reasons for the discrepancy were not discussed. As northern squawfish lapilli were somewhat smaller than sagittae and generally had narrower, more compact daily increments, we may have failed to detect some increments. Additional increments in the lapilli might have been observed using other magnification techniques, such as scanning electron microscopy (Jones 1986).

Intercept values of increment counts regressed on known ages in days for both sagittae (0.54) and lapilli (0.53) were significantly greater than zero in our study, indicating the presence of increments in otoliths from some fish assigned an age of 0. Three patterns of deposition were observed in otoliths of larvae assigned an actual age of 0: no increment present, a marginal or incomplete increment, or a single daily increment. This variation is likely due to our sampling periodicity and the hatching times of individual larvae (hatching occurred over a 2-day period), rather than formation of embryonic increments in northern squawfish otoliths. For this reason, a downward adjustment of ages estimated from larval northern squawfish otolith increment counts is not warranted.

Although we found good correspondence between sagittal increment counts and the true ages of larval northern squawfish, we excluded a high percentage of otoliths (predominantly from fish >10 days old) from the analysis because of increment count disagreements among readers. Disagreement among increment counts was primarily due to difficulty in interpretation of the transition zone in otoliths from older fish. This transition zone of low-contrast material began after 8–11 daily increments had been deposited in both sagittae and lapilli. The increments within this zone were narrow and very weakly expressed and were thus extremely difficult to discern. The temporal position of the transitional zone coincided with the onset of exogenous feeding and largely encompassed the remaining period of yolk sac absorption. Following yolk sac depletion (20 days after hatching), increments widened and were again more clearly defined. Transitional zones have also been observed in

other larval fish otolith studies and have been attributed to a number of developmental processes including metamorphosis, yolk sac absorption, and notochord flexion (Campana 1984a, Hoff et al. 1997).

Some of the reading difficulties and high otolith rejection rates we encountered may be explained by rearing conditions in the laboratory. Laboratory temperature regimes, in particular, may have affected otolith microstructure by influencing larval northern squawfish feeding performance, metabolism, and growth. Fishes with slow growth rates, for example, can have compressed or narrow daily otolith increments that are difficult to discern (Campana 1983, Campana and Neilson 1985, DiCenzo and Bettoli 1995). Growth of northern squawfish in our study may have been impeded by relatively cool (16°C) laboratory rearing temperatures. Although within the 12–18°C range reported for northern squawfish spawning (Beamesderfer 1992), laboratory temperatures may have been substantially cooler than those experienced by wild larvae. Larval northern squawfish develop in shallow shoreline habitats of the Columbia River where temperatures commonly exceed 20°C (Barfoot et al., in press). Increment contrast can also be enhanced by diel temperature fluctuation (Campana 1984a, b; Neilson and Geen 1984; Bestgen and Bundy 1998) which was minimal in this study, but is higher in the natural environment.

Our results indicate that aging wild-caught northern squawfish larvae using daily otolith increment counts is possible. Estimated ages could be used to determine early life history parameters such as distribution of hatching dates and timing of larval drift.

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FIRST OBSERVATIONS OF VERMILION ROCKFISH COURTSHIP ARE FROM A HARVEST REFUGE

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We videotaped courtship displays of vermilion rockfish, *Sebastes miniatus*, on a single reef in the Point Lobos Ecological Reserve, Monterey County, California (Fig. 1). Three courtship sequences involving 6 different fish took place between 1100 and 1500 hours on 28 October 1997 and another sequence occurred at 1145 hours on 24 June 1998. From a literature review, observations of rockfish courtship and mating are very rare and our observations are unique.

Vermilion rockfish are common from the San Benito Islands, Baja California, to Vancouver Island, British Columbia (Miller and Lea 1972) and occur at depths from near-surface to approximately 275 m (Love 1991). They are bottom-dwelling, are typically associated with rocky reefs, and are considered territorial. As is common for rockfishes, the natural mortality rate of vermilion rockfish is relatively low. They are slow to mature sexually – only 50% are mature at 8 years (VenTresca[1] 1992) – and they reproduce through internal fertilization. Studies on vermilion rockfish reproductive seasonality (from examination of gonads) suggest that mating occurs over a broad period from approximately July (Wyllie Echeverria 1987) through November (VenTresca[1] 1992).

Courtship observations were serendipitous and coincidental to our work to collect fish length data using in situ laser-scaled videographic methods (Gingras et al. 1998). Each observation was brief (26–142 seconds). Although we did not observe copulation, the sequences are distinctly different from agonistic behaviors of rockfishes that we have observed and those described by Haaker (1978) and Shinomiya and Ezaki (1991). We did not capture any of the fish and attributed gender based on consistent and differential size, behavior, and color patterns and/or the presence of an extended urogenital papilla, characteristic only of male rockfishes (R.N. Lea, California Department of Fish and Game, personal communication; Helvey 1982; Shinomiya and Ezaki 1991).

Videographers worked 1–5 m from the fish, and had little apparent effect on fish behavior. Courtship displays were generally within 2 m of the rocky or boulder-strewn bottom. The giant kelp, *Macrocystis pyrifera*, canopy was dense in October and sparse during June; the *Pterygophora californica* understory ranged from moderate to sparse. The sea state was calm, underwater horizontal visibility

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[1] VenTresca, D.A. 1992. Vermilion rockfish. Pages 123-124 *in*: W.S. Leet, C.M. DeWees, and C.W. Haugen, editors. California living marine resources and their utilization. Sea Grant Extension Publication UCSGEP-92-12, Davis, California,USA.

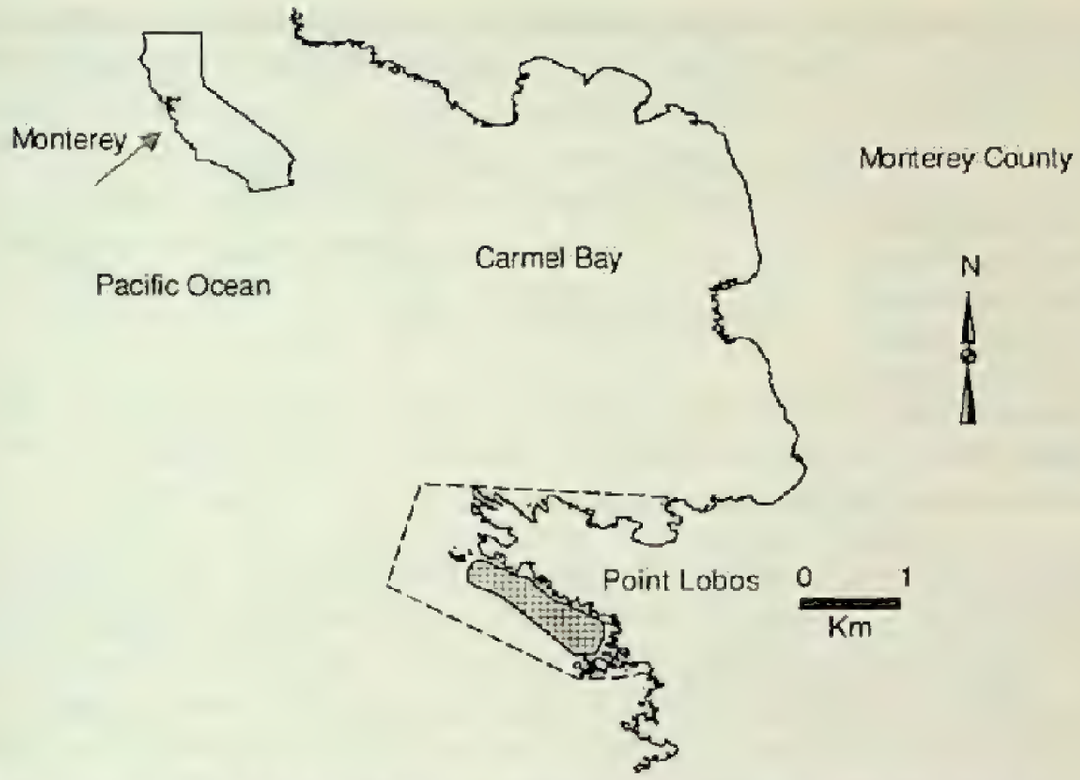

Figure 1. Site map depicting the Point Lobos Ecological Reserve (dashed lines) in southern Carmel Bay, Monterey County, California, and the reef (shaded area) where vermilion rockfish courtship was observed.

was approximately 10 m, and we detected no current. Sea surface temperature was approximately 17°C during the October observations and 12.8°C during the June observation (11.9°C at 22 m). From a rapid, measured-distance (Ugoretz et al. 1997) in situ survey of the density of several rockfishes on this reef during September 1997, the mean density of subadult and adult vermilion rockfish was 1.29/100 m^2 (SE = 0.24).

Two fish were involved in each courtship sequence, although a conspecific was within 4 m of the courting fish during the most recent observation. Each male was substantially larger, and thus older, than the courted female (Phillips 1964, Echeverria and Lenarz 1984). Males usually displayed an extended papilla and often displayed erected pectoral and pelvic fins (Table 1). Females appeared distinctly lighter and more blotchy than males.

Lateral oscillation and lateral display (Shinomiya and Ezaki 1991) were characteristic male courtship behaviors. Lateral oscillation is a very conspicuous side-to-side rolling motion about the long axis of the body (up to ±60°) with a period of 2–5 seconds. A male oscillated laterally when swimming parallel and relatively close to a female. Lateral display was a conspicuous maneuver that brought the ventral surface of a male very close to the snout of a slowly swimming female and only occurred when a male could approach on a near-parallel course and swim abreast of the female ("front position" sensu Helvey 1982). If successful in gaining front position, the male would slowly turn in front of the female.

Females generally swam away or slowly altered course in reaction to attempts by the male to move abreast or in front of them. In 1 courtship sequence, the female

Table 1. Characteristics of pairs of vermilion rockfish observed courting on 28 October 1997 (sequences 1–3) and 24 June 1998 (sequence 4) in the Point Lobos Ecological Reserve, Monterey County, California. y = yes, n = no.

	Sequence			
Characteristics	1	2	3	4
♂ Fork length (mm)	512	478	489	533
♀ Fork length (mm)	418	428	416	437
♂ Extended papilla	y	y	n	y
♂ Lateral oscillation	y	y	y	y
♂ Lateral display	n	y	n	y
♂ Flared fins	y	y	y	y

swam rapidly away from the male after approximately 2 minutes. The male followed for approximately 3 m, then swam slowly in the direction from which he had come. In another sequence, after turning slowly away from the male as he attempted to gain front position, the female turned toward the male and made a rapid vertical ascent with the male following. In the only sequence where a female showed apparent interest in a male, the female descended toward the male from behind and remained motionless while the male laterally displayed (the snout of the female was within approximately 20 cm of the male's extended papilla).

The courtship behaviors we observed are generally similar to those described for *S. inermis* (a bottom-dwelling, generally solitary, and territorial species; Shinomiya and Ezaki 1991) and blue rockfish, *S. mystinus* (a midwater, schooling species). *Sebastes inermis* courtship begins when a female enters a male's territory. Lateral display by the male is typical, and copulation occurs during a brief vertical ascent (Shinomiya and Ezaki 1991). During the June observation, the female appeared to be courted after entering a male's territory, but we have no specific information on vermilion rockfish territories in the Point Lobos Ecological Reserve. The lateral displays we observed were prominent, but lacked the mouth-gaping and body-quivering seen in *S. inermis*. The rapid vertical ascent of a courting pair that we observed appeared similar to the copulatory ascent in *S. inermis*. Courtship in blue rockfish generally involves more than 1 male (Helvey 1982) and occurs in mid-water. As with *S. inermis* and vermilion rockfish, lateral display (although with tail fanning) is prominent. The lateral oscillation behavior we observed has not been described in rockfishes, but has been described in surfperch courtship (M. Cummings, University of California, Santa Barbara, personal communication).

Although no systematic effort has been made to characterize the vermilion rockfish population in California, abundance and mean length in subtidal habitat appear much lower where there is fishing pressure. We suspect that the absence of published descriptions of vermilion rockfish mating and courtship is due to the scarcity of mature individuals in habitat shallow enough to allow routine observation. The density of subadult and adult vermilion rockfish, and several other rockfishes (e.g., copper rockfish, *S. caurinus* and gopher rockfish, *S. carnatus*) on the reef where we observed vermilion rockfish courtship is exceptionally high compared to densities

from similar habitat in the Monterey Bay area and on the Big Sur coast (VenTresca, unpublished data). The differences are likely attributable to a prohibition (dating from 1973) on the harvest of finfish in the Point Lobos Ecological Reserve (McArdle[2] 1997). The unusual population characteristics of rockfishes from this reef should be useful in ecological and fishery studies and in more complete studies on courtship and mating in vermilion rockfish.

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HOMING BEHAVIOR OF A SEVENGILL SHARK RELEASED FROM THE MONTEREY BAY AQUARIUM

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Sevengill sharks, *Notorynchus cepedianus*, have been displayed in the Monterey Bay Habitats exhibit (approximately 1.2 million liters) since the opening of the Monterey Bay Aquarium in 1984. Growth in captivity of sevengill sharks, kept on display for periods up to 2,190 days, has been studied by aquarium staff and volunteers (Van Dykhuizen and Mollet 1992).

On 23 July 1990, a large, female sevengill shark (total contour length = 2.98 m, estimated weight = 112–135 kg) was collected in Humboldt Bay, California by aquarium staff and a commercial fisherman, K. Bates. The sevengill shark was transported to the Monterey Bay Aquarium in an oxygenated transport tank and kept in captivity for 4 years. It was released on 16 June 1994 due to abrasions on the snout that had developed from collisions with aquarium windows and artificial reefs in the Monterey Bay Habitats exhibit. The sevengill shark was tagged at the base of the dorsal fin with a stainless steel dart tag (Floy FH-69, Floy Tag & Mfg., Inc.[1], Seattle, Washington, USA) prior to release in Monterey Bay, California.

On 10 October 1996, this sevengill shark was recaptured in Humboldt Bay, 503 km north of the release site, by C. Brewer, a recreational fisherman. The shark had been at liberty for 845 days and was 2.95 m total length and 125 kg when recaptured. Its reproductive tract contained approximately 100 eggs (1 cm diameter) in the ovary and no embryos in the uterus. The snout was healed, though not pigmented, indicating that sevengill sharks may be successfully reintroduced to the wild after long periods in captivity.

The sevengill shark was attacked just prior to being landed by a larger, unidentified shark which left a 25-cm-wide bite mark on the ventro-lateral surface anterior to the dorsal fin. The bite mark was indicative of the tooth pattern of a white shark, *Carcharodon carcharias*.

A tag and recapture study on sevengill sharks in South Africa (Ebert 1996) reported great distances traveled by tagged sharks (up to 539 km) and long periods at liberty before recapture (>2,503 days); however, there was no correlation between the length of time at liberty and the distance traveled before recapture. Ebert (1996) also reported a large sevengill shark (approximately 3 m total length) that was recaptured at the site of tagging after approximately 730 days at liberty. The homing behavior of the sevengill shark released from the Monterey Bay Aquarium supports the hypothesis

[1] Use of trade names does not imply endorsement by the California Department of Fish and Game.

by Ebert (1996) that sevengill sharks are socially complex animals that return to the same breeding grounds, such as Humboldt and San Francisco bays, where they are abundant during spring and summer (Gotshall et al. 1980).

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We thank K. Bates for assisting in the original capture of the sevengill shark and C. Brewer for reporting its recapture. We are indebted to S. Van Sommeran of the Pelagic Shark Research Foundation for providing the shark tag and P. Collier of the California Department of Fish and Game for gathering information on the fish at recapture.

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A RANGE EXTENSION FOR THE VOLCANO BARNACLE, *TETRACLITA RUBESCENS*

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The biogeographical range of the volcano barnacle, *Tetraclita rubescens*, is reported to extend from Cabo San Lucas, Baja California, Mexico (22°30'N) to San Francisco Bay, California, USA (37°30'N) (Newman and Abbott 1980). However, in 2 surveys of middle and upper rocky intertidal communities in central and northern California during July 1995 (Connolly et al. 1996) and June 1996 (Connolly and Roughgarden 1998), we found this species at 4 of 8 sites surveyed between San Francisco and Cape Mendocino (Table 1). The protocol for both surveys was identical, and is described in detail in Connolly and Roughgarden (1998). The volcano barnacle was sufficiently rare that it was not sampled in transects, so we report its presence separately here. These observations amend a range extension to 39°N proposed earlier for this species (Kinnetic Laboratories[2] 1985). This constitutes a total increase in range of 3° latitude (about 330 km), to 40°24'N, (a 20% increase in latitudinal

Table 1. Northern California sites surveyed during 1995 and 1996.

Site	Location	Year(s) surveyed	Volcano barnacle present?
Cape Mendocino	40°24'N	1995, 1996	Yes
Westport	39°36'N	1995, 1996	Yes
Laguna Point	39°29'N	1995	No
Cabrillo Point	39°21'N	1995	No
Point Arena	38°57'N	1995	Yes
Saunders' Reef	38°52'N	1996	No[a]
Gualala Point	38°45'N	1995	No
Salt Point	38°33'N	1995	Yes

[a]A survey team noted the presence of volcano barnacles at this site in 1984 (Kinnetic Laboratories[2] 1985).

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[2] Kinnetic Laboratories, Inc. 1985. Field survey plan for successional and seasonal variation of the central and northern California rocky intertidal communities as related to natural and man-induced disturbances. Prepared for Minerals Management Service, Pacific OCS Region, U.S. Department of the Interior, Los Angeles, California. Contract No. 14-12-0001-30057. Available from Kinnetic Laboratories, Inc., Santa Cruz, California. Report No. KLI-R-85-2.

range) relative to that reported by Newman and Abbott (1980), and 1.5° latitude (about 165 km; a 10% increase) after accounting for the range extension noted earlier (Kinnetic Laboratories[2] 1985).

Tetraclita rubescens is easily identifiable because its test is composed of 4 plates, whereas all other acorn barnacles in this region have 6 plates (Newman and Abbott 1980, Smith and Carlton 1989). In addition, it is 1 of only 5 acorn barnacles with reddish coloration, the others being *Megabalanus californicus*, *Balanus pacificus*, *B. trigonus*, and *B. amphitrite* (Smith and Carlton 1989). However, these species lack the thatched appearance of *T. rubescens* (Newman and Abbott 1980). Finally, *T. rubescens* is the only one of these species that extends into the middle intertidal zone (Newman and Abbott 1980), where most of these individuals were found.

Barry et al. (1995) proposed that a gradual warming of the nearshore waters of California over the last several decades has led to the northward expansion of species whose northern limits are in or near central California. The range extension reported here supports that hypothesis. However, because *T. rubescens* was present in such small numbers at most sites (e.g., only 1 individual was found at Westport and Cape Mendocino), we cannot rule out the possibility that it has been present north of San Francisco for some time, but has gone undetected until now.

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IN MEMORIAM

ELDEN H. VESTAL (1914–1998)

Elden H. Vestal died on 9 September 1998 at the age of 84 in Napa, California. Elden's career as a fishery biologist with the California Department of Fish and Game spanned 5 decades and was highlighted by the role he played in saving Mono Lake and the survey of 640 Sierra Nevada waters, 130 of which he named.

After he received B.A. and M.A. degrees in zoology from the University of California, Berkeley in 1934 and 1936, he began his career in 1938 as the 5th member of the newly formed Inland Fisheries Biological Staff of the Bureau of Fish Conservation. Following initial work with California Trout Investigations in the Inyo-Mono area, he assisted in coastal salmon and steelhead migration studies in the Eel River basin. From 1939 to 1950, interrupted by World War II, he served as the District Fishery Biologist the Inyo-Mono area. It was his meticulous notes and records from the Rush Creek Test Stream Project in 1947–1951 that helped anchor successful litigation in 1990–1991 to restore flows in Mono Lake tributaries and save Mono Lake from further desiccation.

He became a Regional Fishery Biologist in Fresno (Region 4) in 1951, where his work was dominated by water projects and their impacts on fish and wildlife resources. There were successes and failures in efforts to minimize these impacts. While an adverse water rights decision doomed spring-run chinook salmon and other fishery resources in the San Joaquin River below Friant Dam, 5 years of multi-agency efforts were successful in restoring flows for aquatic life in the 26 km of the Kern River above Kernville.

In 1966, he moved to the San Francisco Bay area as Fisheries Management Supervisor for Region 3. There, he coordinated and supervised inland, coastal, and bay-estuarine fisheries investigations and management; hatchery production and stocking; habitat maintenance and improvement; and the increasing fisheries resource impacts of water developments. An added pleasure was the supervision of a study and recovery plan for the endangered Santa Cruz long-toed salamander at Valencia Lagoon, near Aptos. He retired in 1978 after 41 years of service to the State of California.

He is credited with a number of firsts in California fisheries management. These include chemical fertilization in Sierra Nevada lakes; chemical control of aquatic plants; use of explosives in aquatic plant control; co-planning and direction of initial aerial fish planting in the Sierra Nevada; and initial transplants of endangered Piute cutthroat trout into barren tributaries of their native streams. At the time of his death, he was working with the Natural Resources Defense Council and other agencies in efforts to restore water releases for salmon and other fish and wildlife in the San Joaquin River below Friant Dam.

Mr. Vestal produced some 89 reports and special articles. He co-authored *Mammoth Lakes Sierra–A Handbook for Roadside and Trail*, first published by the Sierra Club in 1959 and revised with a 5th edition in 1989.

He was the recipient of the 1992 Conservation Achievement Award by the California-Nevada Chapter of the American Fisheries Society and, in 1993, the Robert L. Borovicka Conservation Achievement Award by the Western Division of the American Fisheries Society.

Memberships and affiliations included the American Fisheries Society, the Audubon Society, the Mono Lake Committee, and The Nature Conservancy. He was an avid and skilled fly-fisherman and an extraordinarily knowledgeable naturalist and outdoorsman.

He is survived by his beloved wife, Mary, of Napa, whom he met at June Lake and married in 1940; his son and daughter-in-law, Drs. Robert and Bonita Vestal; 2 grandchildren, Zachary and Sarah Vestal; and 2 sisters, Marjorie Sorich and Thelma Vestal.

–Robert E. Vestal, M.D.
1905 Montclair Drive
Boise, Idaho 83702

REFEREE ACKNOWLEDGMENTS

The following individuals have reviewed manuscripts for *California Fish and Game* during 1998 or manuscripts that were published in 1998. The list is as complete as possible. The editors apologize for any omissions and sincerely thank all those who have contributed to the quality of the journal by providing competent and timely reviews.

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